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(54) AMORCES D'OLIGONUCLEOTIDES POUR LA DETECTION SPECIFIQUE PAR PCR DE L'ADN D'ESPECES D'ENTEROBACTERIACEAE PAR L'UTILISATION DE MATRICES DES GENES RFE-RFF

(54) OLIGONUCLEOTIDE PRIMERS FOR THE SPECIFIC PCR DETECTION OF ENTEROBACTERIACEAE SPECIES DNA USING RFE-RFF GENE TEMPLATES

(57) Oligonucleotide primers were designed for the PCR-based detection of rfe-rff genes involved in the biosynthesis pathway leading to the production of enterobacterial common antigen (ECA). E. coli DNA was detected, using specific rfe (WecA) and rffT (WecF) gene primers. Moreover, rffT primers allowed the detection of most members of the Enterobacteriaceae family in biological fluids such as blood and urine with sensitivity as low as 120 bacteria per ml of water. Thus, these primers represent an important step in the molecular diagnosis of major Enterobacteriaceae infections and routine testing of contamination in drinking water and food. The invention relates to particular oligonucleotides, the corresponding primers, the use of these primers for PCR detection of Enterobacteriaceae species, the related method for PCR detection and the related diagnostic assay.

ABSTRACT

Oligonucleotide primers were designed for the PCR-based detection of *rfe-rff* genes involved in the biosynthesis pathway leading to the production of enterobacterial common antigen (ECA). *E. coli* DNA was detected, using specific *rfe* (*Wec*A) and *rffT* (*Wec*F) gene primers. Moreover, *rff*T primers allowed the detection of most members of the *Enterobacteriaceae* family in biological fluids such as blood and urine with sensitivity as low as 120 bacteria per ml of water. Thus, these primers represent an important step in the molecular diagnosis of major Enterobacteriaceae infections and routine testing of contamination in drinking water and food. The invention relates to particular oligonucleotides, the corresponding primers, the use of these primers for PCR detection of *Enterobacteriaceae* species, the related method for PCR detection and the related diagnostic assay.

Oligonucleotide primers for the specific PCR detection of *enterobacteriaceae* species DNA using rfe-rff gene templates

FIELD OF THE INVENTION

The present invention relates to novel PCR oligonucleotide primers for the detection of Enterobacteriaceae species. More particularly, these oligonucleotide primers are directed to *rff*T gene of E*nterobacteriaceae* species. The present invention also relates to uses of these oligonucleotide primers for PCR detection of E*nterobacteriaceae* species and methods for detecting E*nterobacteriaceae* species by PCR using these oligonucleotide primers.

BACKGROUND OF THE INVENTION

Enterobacteriaceae are gram-negative bacilli usually found in the gastrointestinal tract and are frequently associated with septicemia, meningitis, pneumonia, peritonitis and urinary tract infections. This family of bacteria includes *Escherichia coli, Klebsiella pneumoniae, Enterobacter sp., Serratia sp., Salmonella sp., Shigella sp., Citrobacter sp., Yersinia sp., Proteus sp.*, and *Providencia sp.* (Farmer, J.J. *Enterobacteriaceae* Introduction and Identification. *In* Murray P.R. et al.,Manual of Clinical Microbiology 1995, pp. 438-464). A specific common feature of this family (Kuhn, H.M. et al., ECA, the Enterobacterial Common Antigen. FEMS Microbiology Reviews. 1988, 54 , pp. 195-222) is the synthesis of enterobacterial common antigen (ECA).

ECA, a glycophospholipid surface antigen of the *enterobacteriaceae* family, is composed of carbohydrates linked to L-glycerophosphatidyl residues. The carbohydrates comprise N-acetyl-D-glucosamine (GlcNAc), N-acetyl-D-mannosaminuronic (ManNAcA), 4-acetamido-4 and 6-dideoxy-D-galactose (Fuc4NAc). These amino-sugars constitute linear polysaccharide chains of repetitive trisaccharide units. The polysaccharide chains and the lipid component are respectively responsible for serological specificity and anchorage of ECA to the

external membrane of *enterobacteriaceae* (Meier-Dieter U. et al., Nucleotide Sequence of the *Escherichia coli rfe* Gene Involved in the Synthesis of Enterobacterial Common Antigen. J. Biol. Chem. 1992 , 267 , pp. 746-753; and Ohta M.O. et al., Cloning and expression of the *rfe-rff* Gene Cluster of *Escherichia coli*. Mol. Microbiol. 1991, 5, pp. 1853-1862). ECA has immunologic properties (Peter, H. et al. Monoclonal Antibodies to Enterobacterial Common Antigen and to Escherichia coli Lipopolysaccharide Outer Core: Demonstration of an Antigen Determinant Shared by Enterobacterial Common Antigen and E. coli K5 Capsular Polysaccharide. Infect. Immun. 1995, 50, pp. 459-466). A protective action has not been demonstrated in humans but its immunologic properties have been used for diagnostic purposes.

The most frequent diagnostic methods of ECA detection rely on enzyme immunoassay and the passive hemagglutination test (Hübner I. et al. Rapid Determination of Members of the Family E*nterobacteriaceae* in Drinking Water by an Immunological Assay Using a Monoclonal Antibody Against Enterobacterial Common Antigen. Appl. Environ. Microb. 1992, 58, pp. 3187-3191; and Bayardelle P. and Ranger S. Rapid Detection of E*nterobacteriaceae* in Urine with Passive Hemagglutination Test. American Society for Microbiology, 95th Meeting. Washington. May 1995). The Sensitivity of the enzyme immunoassay is low for E*nterobacteriaceae* and varies according to the ECA concentration of the species. The detection limit is about $3,9X10^5$ colony-forming units per ml (CFU/ml) of water and is higher for other species ($1,3X10^7$ CFU/ml). Although specific, the test presents false positive in 0.3% of samples. Passive hemagglutination can be used for urine samples with 100,000 CFU/ml but gave false positive results at 4.6%.

Although ECA genes have been well characterized (Meier-Dieter U. et al. J. Biol. Chem. 1992, 267, pp. 746-753; Ohta M.O. et al. Mol. Microbiol. 1991, 5, pp. 1853-1862; Marolda C.L. and Valvano M.A. Genetic Analysis of the dTDP-Rhamnose Biosynthesis Region of the *Escherichia coli* VW187 (07:KI) *rfb* Gene Cluster: Identification of Fonctional Homologs of *rfbB* and *rfbA* in the *rff* Cluster and Correct Location of the *rffE* Gene. J. Bacteriol. 1995, 177, pp. 5539-5546; Alexander D.C. and Valvano M.A. Role of the *rfe* Gene in the Biosynthesis of the *Escherichia coli*

07-Specific Lipopolysaccharide and Other O-Specific Polysaccharides Containing N-Acetylglucosamine. J. Bacteriol. 1994, 176, pp. 7079-7084; Whitfield C. et al. Modulation of the Surface Architecture of Gram-negative Bacteria by the Action of Surface Polymer: Lipid A-core Ligase and by Determinants of Polymer Chain Length. Mol. Microbiol. 1997, 23, pp. 629-638; Amor P.A. and Whitfield. Molecular and Functional Analysis of Genes Required for Expression of Group IB K Antigens in *Escherichia coli*: Characterization of the his-region Containing Gene Clusters for Multiple Cell-surface Polysaccharides. Mol. Microbiol. 1997, 26 pp.145-161) and new nomenclature implemented (Reeves P.R. et al. Bacterial Polysaccharide Synthesis and Gene Nomenclature. Trends in Microbiology. 1996, 4, pp. 495-503), no PCR-based diagnostic test has yet been developed. Some previous PCR primers designed to determine the function of certain genes are species-specific (Marolda C.L. and Valvano M.A. J. Bacteriol. 1995, 177, pp. 5539-5546). In the new nomenclature, *rfe* and *rff* gene cluster is now identified wec gene cluster.

The detection of pathogen-specific genomic DNA was attempted several years ago from diverse infectious agents in clinical specimens (Song J.H et al. Detection of *Salmonella typhi* in the Blood of Patients with Typhoid Fever by Polymerase Chain Reaction. J.Clin. Microbiol. 1993, 31, pp. 1439-1443; Zhang Y. et al. Detection of *Streptococcus pneumoniae in* Whole Blood by PCR. J. Clin. Microbiol. 1995, 33, pp. 596-601; Greisen,K. et al. PCR Primers and Probes for the 16S rRNA Gene of Most Species of Pathogenic Bacteria, Including Bacteria found in Cerebrospinal fluid. J. Clin. Microbiol. 1994, 32, pp. 335-351; Whelen A.C. and Persing D.H. The Role of Nucleic Acid Amplification and Detection in the Clinical Microbiology Laboratory. Annual Review of Microbiology. 1996, 50, pp. 349-373; Hashimoto Y. et al. Development of Nested PCR Based on the Viab Sequence To Detect *Salmonella* typhi. J. Clin. Microbiol. 1995, 33, pp. 775-777). Broad-range PCR primers for 16S rRNA have been used to amplify either gram-positive or gram-negative bacteria but this technique calls for a combination of PCR and Southern blot hybridization which is time-consuming and does not allow specific identification of *Enterobacteriaceae* species (Greisen,K., M. et al. PCR Primers and Probes for the 16S rRNA Gene of Most Species of Pathogenic Bacteria, Including Bacteria Found in Cerebrospinal fluid.

J. Clin. Microbiol. 1994, 32, pp. 335-351; Song J.H et al. Detection of *Salmonella typhi* in the Blood of Patients with Typhoid Fever by Polymerase Chain Reaction. J.Clin. Microbiol. 1993, 31, pp. 1439-1443).

It has been hypothesized that *rfe* and *rff* genes, implicated in the synthesis of ECA, represent good targets for the broad spectrum detection of E*nterobacteriaceae*. The *rfe* gene is essential for ECA synthesis although its precise function has not been determined. It appears to encode for a transferase which catalyzes the synthesis of GlcNAc-pyrophosphorylundecaprol (lipid I), the essential first intermediate lipid involved in ECA synthesis, while the *rff*T gene encodes for a transferase which reassembles Fuc4NAc and ManNAcA, thus completing the repetitive trisaccharide unit of ECA.

It is an object of the present invention to develop a fast PCR-based diagnostic test and to evaluate its sensitivity and specificity as well as its applicability to the routine detection of *enterobacteriaceae* in biological fluids such as patient blood and urine.

SUMMARY OF THE INVENTION

A first aspect of the invention provides an oligonucleotide selected from the group consisting of:

- RFFT11 having the nucleotide sequence of SEQ ID NO. 1;
- RFFT20 having the nucleotide sequence of SEQ ID NO. 2;
- RFFT17 having the nucleotide sequence of SEQ ID NO. 3;
- RFFT21 having the nucleotide sequence of SEQ ID NO. 4;
- RFFT22 having the nucleotide sequence of SEQ ID NO. 5;
- RFFT7 having the nucleotide sequence of SEQ ID NO. 6;
- RFFT8 having the nucleotide sequence of SEQ ID NO. 7; and
- RFFT18 having the nucleotide sequence of SEQ ID NO. 8.

A second aspect of the invention provides an oligonucleotide primer selected from the group consisting of RFFT11 (SEQ ID NO. 1); RFFT20 (SEQ ID NO. 2); RFFT17 (SEQ

ID NO. 3); RFFT21 (SEQ ID NO. 4); RFFT22 (SEQ ID NO. 5); RFFT7 (SEQ ID NO. 6); RFFT8 (SEQ ID NO. 7); and RFFT18 (SEQ ID NO. 8).

A third aspect of the invention provides an oligonucleotide primer comprising the nucleotide sequence of the oligonucleotide selected from the above-defined group or a substantial part thereof.

A fourth aspect of the invention provides an oligonucleotide primer having a nucleotide sequence of about 21 nucleotides being complementary or identical to a sequence of *rff*T gene of *enterobacteriaceae* species where the sequence of *rff*T gene is located between position +31995 and position +32266 of the *rff*T gene.

A fifth aspect of the invention provides a use of a pair of oligonucleotide primers including a forward primer and a reverse primer for PCR detection of *enterobacteriaceae* species, wherein the forward primer is selected from the group consisting of RFFT11 (SEQ ID NO. 1); RFFT20 (SEQ ID NO. 2); RFFT17 (SEQ ID NO. 3); RFFT21 (SEQ ID NO. 4); RFFT22 (SEQ ID NO. 5); and RFFT7 (SEQ ID NO. 6); and the reverse primer is selected from the group consisting of RFFT8 (SEQ ID NO. 7); and RFFT18 (SEQ ID NO. 8).

A sixth aspect of the invention provides a use of an oligonucleotide primer as provided by the above-mentioned third or fourth aspect of the invention, for PCR detection of *enterobacteriaceae* species.

A seventh aspect of the invention provides a method of detection of *Enterobacteriaceae* species comprising the steps of:

a) extracting DNA from a sample;

b) amplifying a fragment of the extracted DNA by PCR using a forward oligonucleotide primer selected from the group consisting of RFFT11 (SEQ ID NO. 1); RFFT20 (SEQ ID NO. 2); RFFT17 (SEQ ID NO. 3); RFFT21 (SEQ ID NO. 4); RFFT22 (SEQ ID NO. 5); and RFFT7 (SEQ ID NO. 6); and a reverse oligonucleotide primer

selected from the group consisting of RFFT8 (SEQ ID NO. 7); and RFFT18 (SEQ ID NO. 8) and

c) detecting the amplified fragment of the DNA.

An eighth aspect of the invention provides a method of detection of *enterobacteriaceae* species by nested PCR comprising the steps of:

a) extracting DNA from a sample;

b) amplifying a fragment of the extracted DNA by PCR using RFFT17 (SEQ ID NO. 3) as forward oligonucleotide primer and RFFT18 (SEQ ID NO. 8) as reverse oligonucleotide primer;

b-1) amplifying a portion of the fragment amplified in step (b), by PCR using RFFT7 (SEQ ID NO. 6) as forward primer and RFFT8 (SEQ ID NO. 7) as reverse primer; and

c) detecting the portion of the DNA amplified in step (b-1).

A ninth aspect of the invention provides a method of detection of E*nterobacteriaceae* species comprising the steps of:

a) extracting DNA from a sample;

b) amplifying a fragment of the extracted DNA by PCR using a forward oligonucleotide primer comprising the nucleotide sequence of the oligonucleotide selected from the group consisting of RFFT11 (SEQ ID NO. 1); RFFT20 (SEQ ID NO. 2); RFFT17 (SEQ ID NO. 3); RFFT21 (SEQ ID NO. 4); RFFT22 (SEQ ID NO. 5); and RFFT7 (SEQ ID NO. 6) or a substantial part thereof; and a reverse oligonucleotide primer comprising the nucleotide sequence of the oligonucleotide selected from the group consisting of RFFT8 (SEQ ID NO. 7); and RFFT18 (SEQ ID NO. 8) or a substantial part thereof; and

c) detecting the amplified fragment of the DNA.

A tenth aspect of the invention provides a diagnostic assay for diagnosing E*nterobacteriaceae* species in a sample. The assay comprising:

- means for extracting DNA from the sample;

- a solution for detecting E*nterobacteriaceae* species DNA from the extracted DNA by PCR, the solution comprising a PCR master mixture and a pair of primers including a forward primer and a reverse primer selected from the primers above-mentioned in the second, third or fourth aspect of the invention where the reverse primer is in a downstream position from the forward primer.

The present invention provides a simple and rapid technique of detection of Enterobacteriaceae species.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a shows the nucleotide sequence of the *rfe* gene and the respective position of the oligonucleotide primers ECA1, ECA2, K1, K6 and KA7.

Figure 1b shows the nucleotide sequence of the *rff*Tgene and the respective position of the oligonucleotide primers RFFT11, RFFT20, RFFT17, RFFT21, RFFT22, RFFT7, RFFT8 and RFFT18.

Figure 2 represents a gel of electrophoresis showing the specificities of the primer sets tested with *E. coli DNA strain* ATCC 25922. Agarose 1.5% gel electrophoresis run with 10 µl of PCR product and stained with ethydium bromide. Lanes: 1, 250 bp ladder of DNA molecular weight markers (Pharmacia Biotech) ; 2, primers ECA1/ECA2 (763 bp) ; 3, primers rffTI7/rffTI8 (178 bp) ; 4, 50 bp ladder; 5, primers KA7/K6 (122 bp); 6, primers KI/K6 (197 bp); 7, 50 bp ladder; 8, primers rffT7/rffT8 (103 bp); 9, primers rffTII/rffT8 (243 bp); 10, primers rffT20/rffT8 (192 bp); 11, primers rffT21/rffT8 (126bp); 12, primers rffT22/rffT8 (110 bp); 13 negative control with master mix without DNA.

Figure 3 represents a gel of electrophoresis showing sensitivity of nested PCR evaluated by serial dilution of *E. coli*. Template primer pairs were studied with the primers RFFT17 /RFFT18 and ECA1/ECA2. Panel A: agarose (1.5%) gel electrophoresis run with 10 µl of PCR product and stained with ethydium bromide. A

110-bp PCR product was detected with dilutions of *E. coli.* and the *rffT7/rffT8* primer pairs. Lane: 1, 50 bp ladder of DNA molecular weight markers; 2, 40,000 bacteria; 3, 7.000 bacteria; 4, 120 bacteria; 5, 8 bacteria; 6, negative control (blank Qiagen without DNA); 7, negative control (master mix without DNA).

Figure 4 represents a gel of electrophoresis showing the specificities of primers RFFT7 and RFFT8 to detect several E*ntobacteriaceae* species. Agarose (1.5%) gel electrophoresis run with 10 µl of PCR product and stained with ethydium bromide. The primers RFFT7 and RFFT8 yield a 103-bp product. Lane: 1, *E. coli*; 2, *Klebsiella pneumoniae*; 3, *Salmonella typhimurium;* 4, *Citrobacter freundii*; 5, *Enterobacter cloacae*; 6, *Proteus mirabilis;* 7, *Providencia rettgeri;* 8, *Serratia marcescens;* 9, *Shigella sonnei;* 10, *Yersinia enterocolitica;* 11, *Pseudomonas aeruginosa;* 12, positive control of *E. coli* using ECA1/ECA2 primers; 13, 14, negative controls (master mix without DNA); 15, 50 bp ladder of DNA molecular weight markers (Pharmacia Biotech).

Figure 5 represents a gel of electrophoresis showing the detection of E*nterobacteriaceae* species in urine samples with the primers RFFT7/RFFT8. Agarose (1.5%) gel electrophoresis run with 10 ul of PCR product and stained with ethydium bromide. A 103-bp product was present only with positive specimens with enterobacteriaceae. Lane: 1., 50-bp ladder of DNA molecular weight markers (Pharmacia Biotech): 2, *Klebsiella pneumoniae; 3-6, E. coli;* 7, *Klebsiella pneumoniae; 8, 9, E. coli; 10, 11, 12, Klebsiella pneumoniae; 13, 14 Enterococcus;* 15, negative control (blank Qiagen without DNA); *16,* negative control (master mix without DNA).

Figure 6 represents a gel of electrophoresis showing the detection of E*nterobacteriaceae* species in blood samples with the primers RFFT7 and RFFT8. Agarose (1.5%) gel electrophoresis run with 10 µl of PCR product and stained with ethydium bromide. These primers yielded a 103-bp product with all positive specimens. Lane: 1, 50-bp ladder of DNA molecular weight markers (Pharmacia Biotech) ; 2, 3, 4, *E. coli*; 5, *Klebsiella pneumoniae*; 6, 7, 8, *E.* coli; 9, 10, 11, negative blood culture specimens; 12, *Klebsiella pneumoniae;* 13, 14, *E. coli*; 15,

negative control with the benzyl alcohol-guanidine hydrochloride method without DNA; *16*, negative control with master mix without DNA.

DETAILED DESCRIPTION OF THE INVENTION

The present invention concerns the detection of the E*nterobacteriaceae* species. The tested species and their origin are listed in Table 1.

TABLE 1. Gram-negative bacilli used as control in this study.

Isolates	**Origin**	
Enterobacteriaceae		
E.coli	*ATCC	25922
Klebsiella pneumoniae	ATCC	13883
Salmonella typhimurium	ATCC	14028
Citrobacter freundii	ATCC	8090
Enterobacter cloacae	ATCC	23355
Proteus mirabilis	ATCC	7002
Providencia rettgeri	ATCC	872292
Serratia marcescens	ATCC	8100
Shigella sonnei	ATCC	25931
Yersinia enterocolitica	ATCC	862196
Pseudomonas aeruginosa	ATCC	27853

*ATCC, American Type Culture Collection.

Several oligonucleotides have been designed on the basis of *rff*T gene as shown in Figure 1b. These oligonucleotides are listed below:

- RFFT11 having the nucleotide sequence of SEQ ID NO. 1;
- RFFT20 having the nucleotide sequence of SEQ ID NO. 2;
- RFFT17 having the nucleotide sequence of SEQ ID NO. 3;
- RFFT21 having the nucleotide sequence of SEQ ID NO. 4;
- RFFT22 having the nucleotide sequence of SEQ ID NO. 5;
- RFFT7 having the nucleotide sequence of SEQ ID NO. 6;
- RFFT8 having the nucleotide sequence of SEQ ID NO. 7; and
- RFFT18 having the nucleotide sequence of SEQ ID NO. 8.

Based on *rfe* gene, other oligonucleotides have been designed as shown in Figure 1a.

All these oligonucleotides have been used as oligonucleotide primers for PCR detection of E*nterobacteriaceae* species. These oligonucleotides listed in Table 2.

TABLE 2. Oligonucleotide primers

Primer designate	Sequence of oligonucleotide primers	Position of amplicon (bp)	Size of amplicon (bp)
Template primers, external			
rfe			
ECA1 (+strand)	5' GGTGTTCGGCAAGCT TTATCTCAG-3'	(643-666)	763
ECA2 (-strand)	5' GGTTAAATTGGGGCTGCCACCACG-3'	(1405-1382)	
Nested primers			
Rfe			
KI (+strand)	5' CTGGGTTATATCTTTGGCTCC-3'	(671-691)	197
K6 (-strand)	5' ATTGCGAGGCTGGTTTGCC-3'	(867-849)	
KA7 (+strand)	5' GCGGCCATTAATGCGTTCAAC-3'	(746-766)	122
Template primers, external			
rffT			
RFFT17 (+strand)	5' GGTAAGCGTCGGCATCTTCTT-3'	(32089-32109)	178
RFFT18 (-strand)	5' AAACAGCCACGCTTTGCTGT-3'	(32266-32247)	
Nested primers			
rffT			
RFFT7 (+strand)	5' CGGCTTAACTCCTACAGTCAG-3'	(32135-32155)	103
RFFT8 (-strand)	5' GAAAGTAGACCACCAGCATCG-3'	(32237-32217)	
RFFT22 (+strand)	5' GCTGTTCCGGCTTAACTCCTA-3'	(32128-32148)	110
Template primers used alone (non nested)			
RFFT11 (+strand)	5' GCAAACGCGTTGCTGATGTAC-3'	(31995-32015)	243
RFFT20 (+strand)	5' TGGAGACCAATC TTACGTGGG-3'	(32046-32066)	192
RFFT21 (+strand)	5' TGCACAACGGCTTTTTGCTG-3'	(32112-32131)	126
RFFT8 (-strand)	5' GAAAGTAGACCACCAGCATCG-3'	(32237-32217)	

As it can be seen in Figure 1b, the primers based on *rff*T gene are located in a region of *rff*T gene between position +31995 and position +32266. Their length vary between 20 and 21 nucleic acids. As it is well known in the field, a primer for PCR may be shorter or longer than 20 or 21 nucleic acids. Thus, it should be understood that primers being longer or shorter than those shown in Figure 1b, are also part of the present invention as well as primers comprising the nucleotide sequence shown in

SEQ ID NO. 1, 2, 3, 4, 5, 6, 7 or 8, or a substantial part thereof. It is also well known in the field that variations of one nucleic acid or a few nucleic acids may not interfere with the capacity of hybridization of the oligonucleotide primer to DNA. Therefore, a substantial part of said nucleotide sequence also encompasses a primer having one or a few nucleic acids different from the nucleotide sequence.

All the above-described oligonucleotide primers directed to the *rff*T gene are found useful for the PCR detection of E*nterobacteriaceae* species, and methods for PCR detection of E*nterobacteriaceae* species using these primers are achieved by the present invention.

As shown in Table 2, RFFT11 (SEQ ID NO. 1), RFFT20 (SEQ ID NO. 2), RFFT17 (SEQ ID NO. 3), RFFT21 (SEQ ID NO. 4), RFFT22 (SEQ ID NO. 5), and RFFT7 (SEQ ID NO. 6) can be used as forward oligonucleotide primers; and RFFT8 (SEQ ID NO. 7) and RFFT18 (SEQ ID NO. 8) can be used as reverse oligonucleotide primers.

In a preferred embodiment of the invention, RFFT7 (SEQ ID NO. 6) and RFFT8 (SEQ ID NO. 7) are respectively used as forward and reverse primers.

In another preferred embodiment of the invention, RFFT22 (SEQ ID NO. 5) and RFFT8 (SEQ ID NO. 7) are respectively used as forward and reverse primers.

In a further preferred embodiment of the invention, RFFT17 (SEQ ID NO. 3) and RFFT18 (SEQ ID NO. 8) are respectively used as forward and reverse primers.

A PCR or a nested PCR may performed to detect E*nterobacteriaceae* species.

In another further preferred embodiment of the invention, a nested PCR is achieved for the detection of E*nterobacteriaceae* species. In such a nested PCR, several combinations of primers can be used. It is a preferred combination to select RFFT17 (SEQ ID NO. 3) and RFFT18 (SEQ ID NO. 8) as template forward and reverse primers for a first amplification, and RFFT7 (SEQ ID NO. 6) and RFFT8 (SEQ ID

NO. 7) as nested forward and reverse primers for a second amplification. Alternatively, RFFT7 (SEQ ID NO. 6) can be replaced by RFFT22 (SEQ ID NO. 5) in the above-mentioned preferred combination.

Detection of *Enterobacteriaceae* species has been achieved in blood samples and urine samples as described herein below.

Among the *Enterobacteriaceae* species, *Escherichia coli, Klebsiella pneumoniae, Enterobacter sp., Serratia sp., Salmonella sp., Shigella sp.*, and *Citrobacter sp.* have been successfully detected accordingly to the present invention as described herein below.

A diagnostic assay for diagnosing *Enterobacteriaceae* species in a sample is also part of the present invention. The assay comprises:

- means for extracting DNA from the sample;
- a solution for detecting *Enterobacteriaceae* species DNA from the extracted DNA by PCR, the solution comprising a PCR master mixture and a pair of primers including a forward primer and a reverse primer selected from the above-mentioned primers according to the invention and where the reverse primer is in a downstream position from the forward primer.

In order to produce highly specific and general PCR detection assays for *E. coli* and Enterobacteriaceae, one set of template primers has been evaluated for each of the targeted genes (*rfe* and *rffT)*. The primer pairs ECA1/ECA2 for *rfe* and rffT17/rffT18 for *rffT* were used for the amplication of *E. coli* genomic DNA. They both presented a DNA band at 763 bp and 178 bp respectively (Fig. 2, lanes 2 and 3). Then, the specificity of several primer pairs with corresponding template pairs has been examined. As expected, all primer sets presented the expected specific bands (see Figure 2, lines 5, 6, 8, 9, 10 and 11).

Sensitivity of amplification has been tested and the results are shown in Figure 3. Using serial dilutions of bacterial *E. coli* suspensions, detection of 190 bacterial cells

has been achieved under the PCR conditions for nested PCR with the *rfe* primers. The template primers were ECAI/ECA2 and the nested primers, KA7 and K6 while with template primers RFFT17/RFFT18 used in combination with the nested primers pair RFFT22/RFFT8, a detection level of the 120 bacterial cells was obtained (see Figure 3).

Specificity of the assay has been tested and the results are shown in Figure 4 and compiled in Table 3. To assess species specificity of the *rfe* specific primer sets, 60 strains of Enterobacteriaceae which included 44 *E. coli* were used for the *rfe* primers pairs. Although primers from the *rfe* region were highly specific for *E. coli* bacterial strains detecting 44 out of 44 strains tested and none of the 16 other Enterobacteriaceae species was detected. With the objective of developing a general Enterobacteriaceae diagnostic assay and due to the very specific detection of *E. coli* only, evaluation of these primers was not pursued.

The research then focused on the evaluation of *rffT* region primer pairs. Each pair of primers was tested against a limited number of species, including Enterobacteriaceae: *E. coli, Klebsiella sp., Enterobacter sp., Salmonella sp., Enterococcus sp., Serratia sp., Shigella sp.* and *Citrobacter sp,* and non-Enterobacteriaceae: Stapylocococcus sp., Pseudomonas sp., to determine the pairs presenting the larger spectrum of Enterobacteriaceae sp. detection with the highest specificity (see Table 3).

Table 3. Species of Enterobacteriaceae detected by different primer pairs

Isolates	Set of primers					
	RFFT7/8	RFFT22/8	RFFT21/8	RFFT20/8	RFFT11/8	RFFT17/18
E .coli	+	+	+	+	+	+
Klebsiella sp.	+	+	+	+	–	–
Salmonella sp.	+	+	+	+	+	+[a]
Enterobacter sp.	+[a]	–	–	–	–	–
Serratia sp.	+	+	+	–	–	–
Shigella sp.	+	+	+	+	+	+
Citrobacter sp.	–/+	–	NT	NT	–	+

A positive sign confirms the presence of a band with the expected size different for each primer pair.

NT; Not tested

[a] A strong non-specific band was also observed.

The primer pairs RFFT7/RFFT8 had the best profile. All others pairs presented a restricted pattern with slight variations. For example, the primers RFFT17/RFFT18 detected a specific 178-bp band, which was particularly strong with *Citrobacter sp.*, in contrast with the other primer pairs studied. Moreover, some primer pairs occasionally presented a non-specific band which was also observed with primer pair RFFT7/RFFT8 using DNA from *Enterobacter sp.*, *Citrobacter sp.* and *Yersinia sp.*. Nonetheless, RFFT7/RFFT8 primer pairs is still presenting the best detection profile, giving a strong and unequivocal 103 bp signal with 6 out of 10 Enterobacteriaceae tested (see Figure 4).

It has been found that additional purification of bacterial genomic DNA by Qiagen columns did not increase the number of bacteria detected. Furthermore, non-Enterobacteriaceae bacterial species such as *Staphylococcus aureus, Staphylococcus epidermidis* and *Enterococcus sp.*, were also studied and did not yield the desired fragment. Of a total of 116 bacterial species tested and comprising 95 Enterobacteriaceae, 10 *Staphylococcus sp.*, 10 *Pseudomonas sp.* and 1 *Enterococcus sp.*, 54 Enterobacteriaceae were positively detected. None of the non-

Enterobacterial species could be detected with these primers and there were no false positives demonstrating 100% specificity for Enterobacteriaceae. Moreover, *Klebsiella sp.*, *Salmonella sp.* and *Shigella sp.* genomic DNA was always amplified, suggesting 100% detectability of these bacteria species (Table 4).

TABLE 4. Microorganisms detected by PCR with the primers RFFT7 and RFFT8

Microrganisms	**Total no. of isolates**	**No. of isolates detected**	**No. of isolates not detected**
Enterobacteriaceae sp.			
E. coli	10	10	0
Klebsiella sp.	10	10	0
Salmonella sp.	10	10	0
Shigella sp.	8	8	0
Enterobacter sp.	10	7	3
Serratia sp.	10	5	5
Citrobacter sp.	10	4	6
Proteus sp.	10	0	10
Providencia sp.	10	0	10
Yersinia sp.	7	0	7
Non-enterobacteriaceae sp			
Staphylococcus sp.	10	0	10
Pseudomona sp.	10	0	10
Enterococcus sp.	1	0	1

Detection of Enterobacteriaceae in patient urine was assessed, and the results are shown in Figure 5 and compiled in Table 5. The primer pair RFFT7/RFFT8, was evaluated for the detection of Enterobacteriaceae sp. in clinical urine samples. A group of 56 urine specimens (51 from Enterobacteriaceae specimens and 5 non-Enterobacteriaceae) was diagnosed by a conventional method (Kloos W.E and Bannerman T.L *Staphylococcus* and *Micrococcus*. In Murray P.R., Baron E.J., Pfaller M.A., Tenover F.C. and Yolken R.H. (ed), Manual of Clinical Microbiology 1995, pp. 282-298). The PCR technique allowed the detection only of samples which were from *enterobacteriaceae* specimens. Moreover using these primers and a non-nested PCR, DNA from 48 of these 51 specimens (94.1%) could be detected by an approach consisting of a longer PCR program for a total of 50 cycles. A 103 bp product was present only with positive specimens of *Enterobacteriaceae* (fig. 5). There was no

false positive amplification with *Pseudomonas sp.*, Gram-positive cocci and 2 negative urine specimens (Table 5).

TABLE 5. Clinical isolates detected by PCR with the primer pair RFFT7/RFFT8 in urine specimens with > 100,000 CFU/mL [a]

Microorganisms	**Total no. of isolates**	**No. of isolates detected**	**No. of isolates not detected**
Enterobacteriaceae			
E. coli.	39	39	0
Klebsiella sp.	8	8	0
Enterobacter sp.	2	1	1
Proteus sp.	2	0	2
Non-*enterobacteriaceae*			
Pseudomonas sp.	1	0	1
Enterococcus sp	3	0	3
Staphylococcus sp.	1	0	1

[a] Colony-forming units (CFU) determined by the 0.001 ml loop quantitative culture method.

Detection of *Enterobacteriaceae* in blood culture specimens with RFFT7/RFFT8 were assessed, and the results are shown in Figure 6. A total of 13 blood cultures were studied by DNA extraction with the benzyl alcohol-guanidine hydrochloride method. Two sets of primers: RFFT7/RFFT8 and RFFT17/RFFT18 were evaluated for the detection of bacteria. For each pair of primers, only the longer PCR program was used. Out of 10 positive blood cultures there were 8 *E. coli* and 2 *Klebsiella pneumoniae. Ten* were found to contain Enterobacterial-specific PCR fragments with 100% sensitivity with RFFT7/RFFT8 while the sensitivity was 90% (9/10) with RFFT17/RFFT18 which did not detect a *Klebsiella pneumoniae* strain. *No* false positives were obtained from the 3 blood culture-negative specimens (see Figure 6).

DESCRIPTION OF THE TECHNIQUES USED IN THE INVENTION

Growth and characterization of bacteria

The bacterial strains analyzed were either obtained from the American Type Culture Collection or isolated in a clinical microbiology laboratory. Gram-negative bacilli were identified with the Vitek system (Mérieux, France). Cocci were characterized by the conventional method of Kloos and Bannerman (Supra). The bacteria were cultured on blood agar plates and incubated at 37^{0}C for 18-24 h. The colonies obtained were inoculated in trypticase soy broth (TSB) and incubated at the same temperature for 18-24 h. Viable counts were achieved by plating serial dilutions of the broth culture.

Preparation of bacterial genomic DNA

DNA from bacterial suspension cultures in TSB was extracted according to the method of LeBouguenec et al. (Le Bouguenec C. et al. Rapid and Specific Detection of the *pap, afa,* and *sfa* Adhesin-Encoding Operons in Uropathogenic *Escherichia* coli Strains by Polymerase Chain Reaction. J. Clin. Microbiol. 1992, 30, pp. 1189-1193). Briefly, bacteria contained in 1 ml of broth culture were collected by centrifugation at 3,000rpm for 3 min. The pellet was re-suspended in 200 µl of sterile distilled water, boiled for 15 min and again centrifuged at 14,000g for 3 min. The supernatant was carefully transferred to a sterile tube and maintained at -20^{0}C.

DNA extraction from urine specimens

Patient urine specimens with 100,000 CFU/ml or more were obtained from the hospital microbiology laboratory and stored at -20^{0}C. DNA was extracted using QiAamp tissue method according to the manufacturer's protocol (QIAGEN, Mississauga, Ontario, Canada) with minor modifications. Briefly, 1 ml of urine was centrifuged at 6,000g for 10 min. The supernatant was discarded, and the remaining pellet was lysed by adding 180 ul of lysis buffer (buffer ATL, provide by manufacturer)

and detergent. 20 µl of proteinase K (20 mg/ml) was also added, followed by incubation at 55°C for 2 h. Subsequently, 200 µl of AL buffer (provide by manufacturer) was added, vortexed vigorously, incubated at 70°C for 10 min and then at 95°C for 15 min, followed by mixing with 210 µl of sterile 100% ethanol. This suspension was applied on a Qiagen column and centrifuged for 1 min in a 2-ml collection tube at 6,000g. The column was successively washed with AW buffer (provided by manufacturer), for 1 and 3 min, and the filtrates were discarded. Two hundred µl of preheated AL buffer (at 70° C) was added to the column. DNA was recovered by 1 min centrifugation and stored in a sterile microfuge tube at -20°C.

Bacterial DNA extraction from blood cultures

Bacterial strains were recovered from blood cultures using BACTEC 9240 instrumentation (Becton Dickinson, Sparks, MD) and standard 10 aerobic/F and lytic/10 anaerobic F bottles. For maximum recovery, a 10-ml aliquot of patient blood was inoculated in each bottle and incubated at 35°C with agitation. A sensor located at the bottom of each bottle responded by increasing fluorescence proportionally to the elevation of CO_2 concentration resulting from organism metabolism (Rohner P. et al. Comparative Evaluation of BACTEC Aerobic Plus/F and Septi-Chek Release Blood Culture Media. J. Clin. Microbiol. 1996, 34, pp.126-129). When a positive signal was obtained, a sample was taken from the bottle with a sterile syringe and Gram-staining was performed to confirm the presence of viable microorganisms. If Gram-staining showed Gram-negative bacteria, subcultures were prepared on MacConkey agar plates and incubated aerobically at 37°C.

Subcultures were also produced on blood and/or chocolate agar plates which were incubated at 37°C in 5% CO_2. The anaerobic subcultures on blood agar were supplemented with vitamin and incubated overnight. For aerobic bacteria, after overnight incubation, if colonies were observed on blood agar plates, identification was undertaken by conventional methods (Farmer, J.J. *Enterobacteriaceae* Introduction and Identification. *In* Murray P.R. et al. Manual of Clinical Microbiology 1995, pp. 438-464; Kloos W.E and Bannerman T.L *Staphylococcus* and *Micrococcus*.

In Murray P.R. et al., Manual of Clinical Microbiology 1995, pp. 282-298). The Gram-negative rods were identified biochemically with the Vitek system.

DNA was extracted only in samples taken from positive aerobic bottles when positive growth was observed, or after 5 days when the culture bottles were discarded. Five hundred µl aliquots of the broth medium containing erythrocytes were removed from the aerobic bottles under sterile conditions, put in conical tubes and kept at -20^{0}C until processed.

The benzyl alcohol-guanidine hydrochloride method (Fredricks D.N. and Relman D.A. Improved Amplification of Microbial DNA from Blood Cultures by Removal of the PCR inhibitors Sodium Polyanetholsulfonate. J. Clin. Microbiol. 1998, 36, pp. 2810-2816) was used for DNA extraction, with minor modifications. A 100-µl aliquot of the blood broth mixture containing bacteria was lysed by brief mixing with the same amount of lysis buffer (5 M guanidine hydrochloride-100 mM Tris, pH 8.0 in sterile water). This was followed by the addition of 400 µl of water and 800 µl of 99% benzyl alcohol (Sigma Chemical Company) and extensive vortexing. The suspension was centrifuged for 5 min at 6,000g, and 400 µl of the supernatant transferred to a microfuge tube for precipitation with 40 µl of 3.0 M sodium acetate and 440 µl of isopropanol. The DNA precipitate was recovered by centrifugation at 6,000g for 30 min, washed with 70% ethanol, air-dried, and dissolved in 100 µl of 10 mM Tris-0.1 mM EDTA, pH 8.5.

Oligonucleotide primers for PCR detection

E. coli genome organization in the ECA operon is known and its complete sequence is available. Based on this knowledge, several primers were designed from the *rfe/rff* group of genes located on *E. coli* genome positions 84.5 to 86.5 min (Daniels D.L. et al. Analysis of the *Escherichia coli* genome: DNA sequence of the region from 84.5 to 86.5 minutes. Science 1992, 257 (5071), pp. 771-778; Blattner F.R. et al. The Complete Genome Sequence of *Escherichia* coli K-12. Science. 1997, 277, pp. 1453-1462). Since nucleotide sequence of the *E. coli rfe* gene have already been

published, we took advantage of this sequence to design several sets of primers (see table 2) for the amplification of the *E coli* DNA (Meier-Dieter U. et al. Nucleotide Sequence of the *Escherichia coli rfe* Gene Involved in the Synthesis of Enterobacterial Common Antigen. J. Biol. Chem. 1992 , 267, pp. 746-753). Their specific positions on the sequence are presented in figure 1a. The sequence of *rff*T gene was available by genebank (accession number : M87049) and the positions of the primers are shown in figure 1b.

The template primers ECA1/ECA2 and RFFT17/RFFT18 were used in combination with nested primers for PCR-based detection of *E. coli* and E*nterobacteriaceae* strains respectively.

PCR Amplification of bacterial DNA

Reactions were performed in a total volume of 55 ul. Forty-five µl of a master mixture containing 5 µl of 10 X PCR buffer (500 mM KCl, 15 mM $MgCl_2$ and 100 mM Tris-HCl), 8 µl of 1.25 µM of dNTP stock solution, 2.5 µl of each of the forward and reverse primers (stock 1 µg/ml) and 0.25 µl (1.25 units) of TAQ polymerase (Pharmacia, Baie d'Urfé, Québec, Canada) and 26.75 µl of sterile distilled water were mixed with 10 ul of bacterial DNA samples. The negative controls contained all ingredients of the master mixture without any DNA samples. For urine specimens, the final concentration of the primers was 0.45 µM, and 5 units of TAQ polymerase were used. For the nested PCR and blood culture specimens, the concentration of the primers and Taq polymerase was respectively 0.9 µM and 2.5 units. A 3 µl aliquot of template PCR reaction product was used for the nested PCR. Amplifications were performed with a Perkin Elmer 9600 cycler under the following conditions: cycles of 2 min denaturation at 94^{0}C and 1 min annealing at 60^{0}C, followed by 2 min of polymerization at 72^{0}C (this cycle was repeated 25 times). For nested PCR, the first amplification (template) cycle was as described previously, while the nested PCR consisted of 25 cycles of shorter duration: 30 sec denaturation at 94^{0}C, 15 sec, annealing at 60^{0}C and 30 sec polymerization at 72^{0}C. A 10 µL aliquot from the PCR reaction product was

electrophoresed on 1.5% agarose mini-gel, stained with ethidium bromide and photographed with a Polaroid camera.

For the study of urine specimens, a non-nested PCR was chosen to avoid contamination, which can occur with the nested PCR, particularly with the transfer of DNA into another tube after the first amplification. The non-nested approach consisted of the successive use of the longer PCR program followed by the shorter one for a total of 50 cycles.

SEQUENCE LISTING

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(ii) TITLE OF THE INVENTION: Oligonucleotide primers for the specific PCR detection
of enterobacteriaceae species DNA using RFE-RFF gene templates

(iii) NUMBER OF SEQUENCES: 8

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER-READABLE FORM:
(A) TYPE OF SUPPORT: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WordPad

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION DATA: 2,286,512
(B) FILING DATE: 27 October 1999

(viii) PATENT AGENT INFORMATION
(A) NAME: ROBIC
(B) REFERENCE NUMBER: 26740-0005

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 nucleic acids
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) ANTISENS: NO

(ix) FEATURE:
(A) NAME/KEY: RFFT11
(B) LOCATION: 31995..32015

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GCAAACGCGT TGCTGATGTA C 21

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 nucleic acids
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) ANTISENS: NO

(ix) FEATURE:
(A) NAME/KEY: RFFT20
(B) LOCATION: 32046..32066

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TGGAGACCAA TCTTACGTGG G 21

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 nucleic acids
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) ANTISENS: NO

(ix) FEATURE:
(A) NAME/KEY: RFFT17
(B) LOCATION: 32089..32109

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GGTAAGCGTC GGCATCTTCT T 21

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 nucleic acids
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) ANTISENS: NO

(ix) FEATURE:
(A) NAME/KEY: RFFT21

(B) LOCATION: 32112..32131

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TGCACAACGG CTTTTTGCTG 20

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 nucleic acids
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) ANTISENS: NO

(ix) FEATURE:
(A) NAME/KEY: RFFT22
(B) LOCATION: 32128..32148

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCTGTTCCGG CTTAACTCCT A 21

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 nucleic acids
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) ANTISENS: NO

(ix) FEATURE:
(A) NAME/KEY: RFFT7
(B) LOCATION: 32135..32155

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CGGCTTAACT CCTACAGTCA G 21

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 nucleic acids
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) ANTISENS: YES

(ix) FEATURE:
(A) NAME/KEY: RFFT8
(B) LOCATION: 32237..32217

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GAAAGTAGAC CACCAGCATC G 21

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 nucleic acids
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) ANTISENS: YES

(ix) FEATURE:
(A) NAME/KEY: RFFT18
(B) LOCATION: 32266..32247

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

AAACAGCCAC GCTTTGCTGT 20

Claims:

1. An oligonucleotide selected from the group consisting of:
- RFFT11 having the nucleotide sequence of SEQ ID NO. 1;
- RFFT20 having the nucleotide sequence of SEQ ID NO. 2;
- RFFT17 having the nucleotide sequence of SEQ ID NO. 3;
- RFFT21 having the nucleotide sequence of SEQ ID NO. 4;
- RFFT22 having the nucleotide sequence of SEQ ID NO. 5;
- RFFT7 having the nucleotide sequence of SEQ ID NO. 6;
- RFFT8 having the nucleotide sequence of SEQ ID NO. 7; and
- RFFT18 having the nucleotide sequence of SEQ ID NO. 8.

2. An oligonucleotide primer having the nucleotide sequence of the oligonucleotide of claim 1.

3. The oligonucleotide primer according to claim 2, wherein the primer is selected from the group of RFFT17 (SEQ ID NO. 3); RFFT22 (SEQ ID NO.5); RFFT7 (SEQ ID NO. 6); RFFT8 (SEQ ID NO. 7); and RFFT18 (SEQ ID NO. 8).

4. An oligonucleotide primer comprising the nucleotide sequence of the oligonucleotide of claim 1 or a substantial part thereof.

5. An oligonucleotide primer having a nucleotide sequence being complementary or identical to a sequence of *rff*T gene of Enterobacteriaceae species where the sequence of *rff*T gene is located between position +31995 and position +32266.

6. An oligonucleotide primer of claim 5, wherein the nucleotide sequence is of about 21 nucleotides.

7. Use of a pair of oligonucleotide primers including a forward primer and a reverse primer for PCR detection of *Enterobacteriaceae* species from a sample, wherein the forward primer is selected from the group consisting of RFFT11 (SEQ ID

NO. 1); RFFT20 (SEQ ID NO. 2); RFFT17 (SEQ ID NO. 3); RFFT21 (SEQ ID NO. 4); RFFT22 (SEQ ID NO. 5); and RFFT7 (SEQ ID NO. 6); and the reverse primer is selected form the group consisting of RFFT8 (SEQ ID NO. 7); and RFFT18 (SEQ ID NO. 8).

8. Use of claim 7, wherein the forward primer is RFFT22 (SEQ ID NO. 5) and the reverse primer is RFFT 8 (SEQ ID NO. 7).

9. Use of claim 7, wherein the forward primer is RFFT7 (SEQ ID NO. 6) and the reverse primer is RFFT8 (SEQ ID NO. 7).

10. Use of claim 7, wherein the forward primer is RFFT17 (SEQ ID NO. 3) and the reverse primer is RFFT18 (SEQ ID NO. 8).

11. Use of an oligonucleotide primer according to claims 4, 5 or 6, for PCR detection of E*nterobacteriaceae* species.

12. Use of any one of claims 7 to 11, wherein the sample is a blood sample or a urine sample.

13. Use of any one of claims 7 to 12, wherein the E*nterobacteriaceae* species is selected from the group consisting of *Escherichia coli, Klebsiella pneumoniae, Enterobacter sp., Serratia sp., Salmonella sp., Shigella sp.,* and *Citrobacter sp.*

14. Method of detection of E*nterobacteriaceae* species comprising the steps of:

a) extracting DNA from a sample;

b) amplifying a fragment of the extracted DNA by PCR using a forward oligonucleotide primer selected from the group consisting of RFFT11 (SEQ ID NO. 1); RFFT20 (SEQ ID NO. 2); RFFT17 (SEQ ID NO. 3); RFFT21 (SEQ ID NO. 4); RFFT22 (SEQ ID NO. 5); and RFFT7 (SEQ ID NO. 6); and a reverse oligonucleotide primer selected from the group consisting of RFFT8 (SEQ ID NO. 7); and RFFT18 (SEQ ID NO. 8) and

c) detecting the amplified fragment of the DNA.

15. Method of claim 14, wherein the forward primer is RFFT7 (SEQ ID NO. 6) and the reverse primer is RFFT8 (SEQ ID NO. 7).

16. Method of claim 14, wherein the forward primer is RFFT 22 (SEQ ID NO. 5) and the reverse primer is RFFT8 (SEQ ID NO. 7).

17. Method of claim 14, wherein the forward primer is RFFT17 (SEQ ID NO. 3) and the reverse primer is RFFT18 (SEQ ID NO. 8).

18. Method of claim 17, wherein step (b) is followed by the step of:

b-1) amplifying a portion of the fragment amplified in step (b), by PCR using a forward primer being RFFT7 (SEQ ID NO. 6) and a reverse primer being RFFT8 (SEQ ID NO. 7).;

and wherein step (c) is detecting the portion amplified in step (b-1).

19. Method of detection of E*nterobacteriaceae* species comprising the steps of:

a) extracting DNA from a sample;

b) amplifying a fragment of the extracted DNA by PCR using a forward oligonucleotide primer comprising the nucleotide sequence of the oligonucleotide selected from the group consisting of RFFT11 (SEQ ID NO. 1); RFFT20 (SEQ ID NO. 2); RFFT17 (SEQ ID NO. 3); RFFT21 (SEQ ID NO. 4); RFFT22 (SEQ ID NO. 5); and RFFT7 (SEQ ID NO. 6) or a substantial part thereof; and a reverse oligonucleotide primer comprising the nucleotide sequence of the oligonucleotide selected from the group consisting of RFFT8 (SEQ ID NO. 7); and RFFT18 (SEQ ID NO. 8) or a substantial part thereof; and

c) detecting the amplified fragment of the DNA.

20. Method of detection of E*nterobacteriaceae* species comprising the steps of:

a) extracting DNA from a sample;

b) amplifying a fragment of the extracted DNA by PCR using a forward oligonucleotide primer having a nucleotide sequence substantially identical to a 5' - 3' sequence of *rff*T gene of E*nterobacteriaceae* species; and a reverse primer having a nucleotide sequence substantially identical to a complementary sequence of the 5'-3' sequence of the *rff*T gene of E*nterobacteriaceae* species wherein the forward primer and the reverse primer or the 5'-3' sequence are complementary sequences of the *rff*T gene located between position +31995 and position +32266; and

c) detecting the amplified fragment of the DNA.

21. Method of claim 20, wherein the primers have a nucleotide sequence of about 21 nucleotides.

22. Diagnostic assay for diagnosing E*nterobacteriaceae* species in a sample, the assay comprising:

- means for extracting DNA from the sample;
- a solution for detecting E*nterobacteriaceae* species DNA from the extracted DNA by PCR, the solution comprising a PCR master mixture and a pair of primers including a forward primer and a reverse primer as defined in claims 7, 8, 9 or 10.

23. Diagnostic assay for diagnosing E*nterobacteriaceae* species in a sample, the assay comprising:

- means for extracting DNA from the sample;
- a solution for detecting E*nterobacteriaceae* species DNA from the extracted DNA by PCR, the solution comprising a PCR master mixture and a pair of primers including a forward primer as defined in claims 4, 5, or 6, and a reverse primer as defined in claims 4, 5, or 6 and being in a downstream position from the forward primer.

Figure 1a (*rfe*)

```
1       GAATTCCTCA TTAATAAACT GGCCATGACC AAGACCAATG ACGATTTCTT CGAAATGATG
61      AAACGCTCAT AAATTTGTCT TATGCCAAAA ACGCCACGTG TTTACGTGGC GGCTTTTATA
121     TCTGTAATCT TAATGCCGCG CTGGCGATGT TAGGAAAATT CCTGGAATTT GCTGGCGTGT
181     TATGCAATTT GCATATCAAA TGGTTAATTT TTGCACAGGA CTGGTGGGTT CGGAACGGAC
241     TTTCCCTTCT GAATAAAGGT CTTAGTGGTT ATACTTCTGC TAATAATTTT CTCTGAGAGC
301     ATGCATTGTG AATTTACTGA CAGTGAGTAT TGATCTCATC AGTATTTTTT TATTCACGAC
361     ACTGTTTCTG TTTTTTGCCC GTAAGGTGGC AAAAAAAGTC GGTTTAGTGG ATAAACCAAA
421     CTTCCGCAAA CGTCACCAGG GATTGATACC TCTCGTTGGG GGGATTTCGG TTTACGCAGG
481     GATTTGCTTC ACGTTCGGAA TTGTCGATTA CTATATTCCG CATGTATCTC TCTATCTCGC
541     TTGTGCCGGT GTGCTTGTTT TCATTGGCGC GCTGGATGAC CGTTTTGATA TCAGCGTAAA
                                                              ECA1→
601     AATCCGTGCC ACCATACAGG CCGCTGTTGG CATTGTTATG ATGGTGTTCG GCAAGCTTTA
                          K1→
661     TCTCAGTAGC CTGGGTTATA TCTTTGGCTC CTGGGAGATG GTGCTCGGAC CGTTTGGTTA
                                                  KA7→
721     CTTCCTGACG CTATTTGCCG TCTGGGCGGC CATTAATGCG TTCAACATGG TTGATGGCAT
781     TGATGGCTTG CTGGGCGGGT TGTCCTGCGT CTCGTTTGCA GCAATCGGTA TGATTTTGTG
                                  ←K6
841     GTTCGACGGG CAAACCAGCC TCGCAATCTG GTGCTTTGCG ATGATCGCCG CCATCCTGCC
901     ATACATCATG CTTAACCTTG GTATCCTGGG TCGCCGCTAC AAAGTCTTTA TGGGTGATGC
961     GGGCAGTACG CTGATTGGTT TTACCGTTAT CTGGATCCTG CTCGAAACGA CCCAGGGCAA
1021    AACCCATCCC ATCAGCCCGG TTACCGCTTT GTGGATAATC GCCATTCCGC TAATGGATAT
1081    GGTGGCGATT ATGTACCGTC GCCTGCGTAA AGGCATGAGC CCATTCTCTC CTGACCGTCA
1141    GCATATTCAC CATTTGATCA TGCGTGCCGG GTTTACTTCC CGTCAGGCGT TTGTGCTGAT
1201    TACCCTTGCC GCAGCACTGC TCGCTTCCAT TGGCGTGCTG GCAGAATATT CTCATTTTGT
1261    CCCGGAGTGG GTCATGCTGG TGCTCTTTTT GCTAGCATTC TTCCTCTATG GATATTGCAT
1321    TAAGCGTGCC TGGAAAGTTG CTCGCTTTAT TAAGCGCGTA AAACGCAGAC TGCGTAGAAA
                        ←ECA2
1381    TCGTGGTGGC AGCCCCAATT TAACCAAATA AATGAGGATG TGATGACACA ACCAATGCCT
1441    GGGAAACCGG CCGAAGACGC TGAAAATGAA CTGGATATTC GTGGGTTGTT TCGTACCTTG
1501    TGGGCTGGGA AGCTATGGAT TATTGGCATG GGGCTGGCGT TTGCGTTAAT CGCGCTGGCG
1561    TATACGTTTT TTGCTCGTCA GGAGTGGAGC TCGACGGCGA TTACCGATCG TCCAACGGTG
1621    AATATGCTGG GGGGATATTA CTCGCAGCAG CAATTTTTGC GTAACCTGGA TGTCCGTTCA
1681    AACATGGCTT CTGCCGACCA ACCATCGGTC ATGGACGAAG CCTATAAAGA GTTTGTTATG
1741    CAACTGGCCT CGTGGGATAC CCGCAGAGAG TTCTGGCTGC AAACCGACTA TTACAAACAG
1801    CGGATGGTGG GCAACAGCAA AGCCGATGCG GCGTTGCTGG ATGAAATGAT TAACAACATC
1861    CAGTTTATCC CCGGAGACTT TACCCGCGCG GTCAATGACA GCGTGAAGCT TATTGCCGAA
1921    ACGCGCCTGA CGCTAATAA
```

Figure 1b (*rffT*):

30601 CTCTTTGTTG TGGCGTGTTT TTACTCTGGC GTAGGCGGGC ATGACTGTAC TGATTCACGT
30661 ACTGGGATCG GATATCCCTC ACCATAACCG AACCGTTTTG CGGTTTTTCA ATGACGCGCT
30721 GGCCGCGACG AGCGAGCACG CGCGCGAGTT TATGGTTGTT GGCAAGGACG ACGGCTTAAG
30781 TGATAGCTGT CCGGCGCTTT CTGTGCAATT TTTCCCTGGA AAAAATCGCT GGCGGAASGC
30841 GGTCATCGCG AAAGCAAAAG CTAACCGTCA GCAGCGTTTT TTCTTCCACG GTCAGTTCAA
30901 TCCCACACTG TGGCTGGCTC TGCTGAGTGG TGGCATTAAG CCCAGCCAGT TTTTCTGGCA
30961 TATCTGGGGG GCAGACCTGT ATGAGCTTTC CAGTGGCTTG AGATATAAGC TTTTTTACCC
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31081 CTTTTTTGCC AAAACGCACC CAAAGGTGCG GGGCGAACTG CTGTTCTTCC CGACGCGGAT
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31741 GTTTGTTGTC TGGCTGCTCT GCACGCTGTT TATTGCCACG CTGACCTGGT TTGAGTTTCG
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31861 CGGCTTCCCG CTGACCAGCG TGCTGGTATT TCGCTTTGAT GTTGGTGTCG CGCCGCCAGA
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RFFT11→

31981 CAAAACCCGC CTACGCAAAC GCGTTGCTGA TGTACCGCGC CGTCCGCTGT TTACCATGAA

RFFT20→ RFFT17→

32041 CCGCGTGGAG ACCAATCTTA CGTGGGTGAT CCTGATGGGT ATCGCGCTGG TAAGCGTCGG

RFFT21→ RFFT7→

32101 CATCTTCTTC ATGCACAACG GCTTTTTGCT GTTCCGGCTT AACTCCTACA GTCAGATCTT

RFFT22→

32161 TTCCAGTGAA GTCTCCGGCG TGGCGTTAAA ACGCTTCTTT TACTTTTTCA TCCCGGCGAT

←RFFT8 ←RFFT18

32221 GCTGGTGGTC TACTTTCTGC GCCAGGACAG CAAAGCGTGG CTGTTTTTCC TCGTCAGCAC
32281 GGTCGCCTTT GGCTTGCTGA CTTATATGAT TGTCGGCGGC ACTCGCGCCA ATATCATCAT
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32401 GGCGGCGGCG GGCGTGCTGG GGATTGTTGG CATGTTCTGG CTGGCACTAA AACGCTATGG
32461 AATGAATGTG AGCGGCGATG AAGCGTTCTA TACGTTTCTC TATCTCACTC GCGACACCTT
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32941 GGTCTTTTTT ATCGTGGTCT TCGGCGCATG TCTGATGATC GCAAAACTGT TGTACTGGCT
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Figure 2:

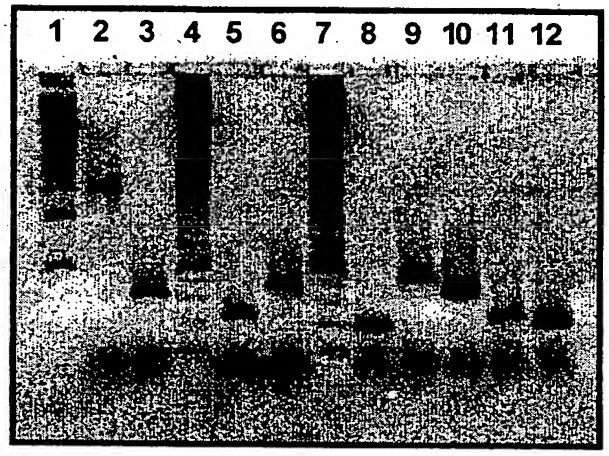

Figure 3:

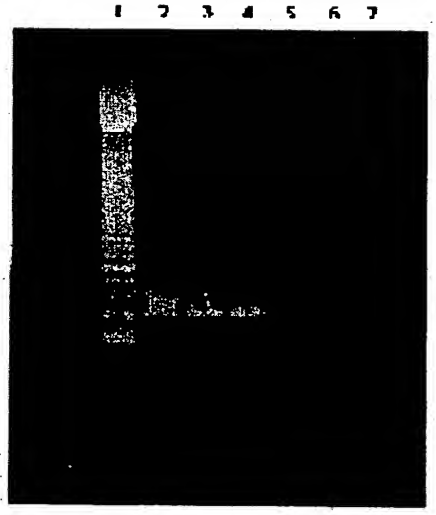

Figure 4:

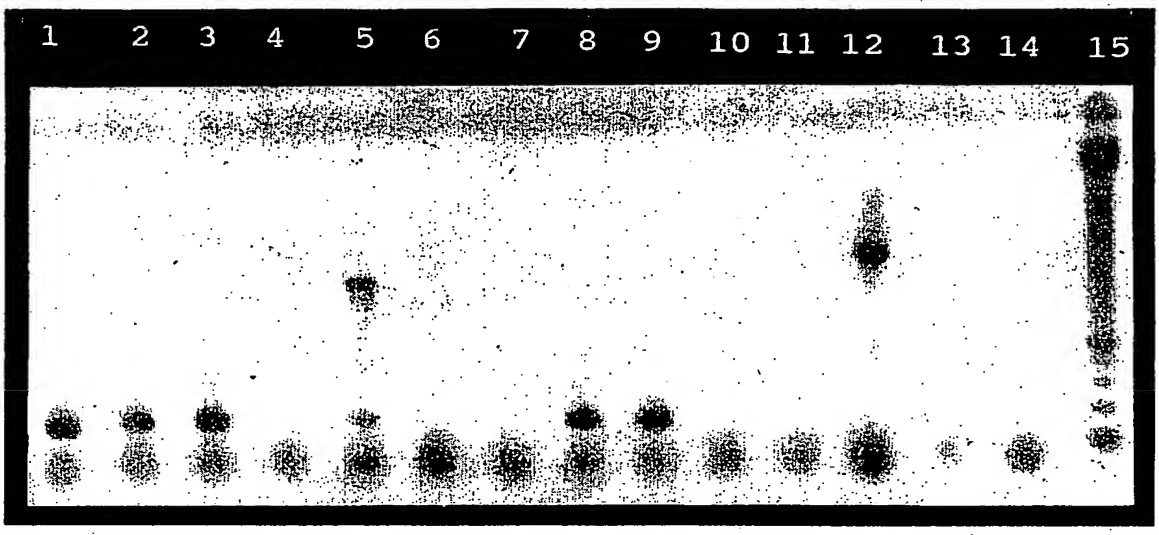

Figure 5:

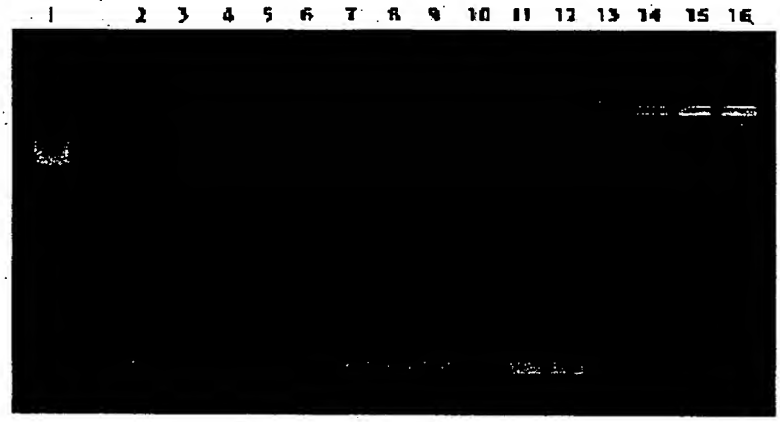

Figure 6: